

ESTROGEN'S EFFECTS ON OXIDATIVE STRESS
LEVELS IN GLIAL CELLS

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A THESIS SUBMITTED TO

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BY

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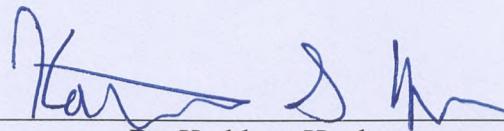
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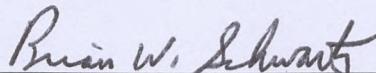


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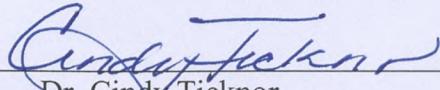


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ABSTRACT

A decrease in astrocyte function and viability has been linked to many neurodegenerative diseases, including schizophrenia, major depressive disorders, Alzheimer's disease, and Amyotrophic Lateral Sclerosis (ALS). Previous research indicates that some estrogens may have a positive effect on neuron and astrocyte viability. Estrogens' effects on the brain are of interest as estrogens are commonly prescribed as hormone replacement therapy to reduce the symptoms of menopause in women. Unfortunately, these effects on are not completely understood. The goal of the project was to discover if estrogen has any protective effect on astrocytes exposed to oxidative stress. We measured the oxidative stress level of N1 1321 glial cells that were exposed to 600 μ M hydrogen peroxide. The astrocytes were pretreated for one hour with one of four types of estrogen at four concentrations ranging from 10nM – 10 μ M. Two endogenous human estrogens, 17 alpha-estradiol and 17 beta-estradiol, and two equine estrogens, equilin and equilenin, were utilized. Only 17 alpha-estradiol showed a statistical difference in the oxidative stress levels of astrocytes. However, the results indicate that differing estrogen concentration still had no effect on the oxidative stress levels in unstressed astrocyte populations.

ACKNOWLEDGEMENTS

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INTRODUCTION

The importance of glial cells in the brain cannot be overstated. Glial cells function to support neurons and comprise as much as 90% of the human brain (He and Sun, 2006). One of the most common glial cells is the stellate-shaped astrocyte cell. Astrocytes have a variety of essential functions in the central nervous system (CNS). As one function, astrocytes sustain neurons by providing glycogen as a source of energy to fuel neurons (Reynolds, 2012).

In addition to providing crucial energy and nourishment, astrocytes are employed in a multitude of other crucial brain functions related to sustainability. Notably, astrocytes are involved in the maintenance of the extracellular ion concentration in the CNS, preserving the permeable membrane known as the blood-brain barrier, and assisting in the repair and regeneration of nervous tissue after an injury (Venkatesh et al., 2013). Astrocytes have also been shown to be involved in neuronal migration by providing a natural scaffolding for neurons (Venkatesh et al., 2013). Additionally, astrocytes have been associated with the reuptake of neurotransmitters and the release of gliotransmitters in the brain (Agulhon et al., 2010). Furthermore, astrocytes have been shown to assist with synaptogenesis by releasing proteins, which help to increase the number of synapses formed between neurons (Barker and Ullian, 2008). Finally, there is even evidence that astrocytes of the subventricular zone may act as neural stem cells. These astrocytes have been shown to be precursor cells to neuroblasts and ultimately new neurons (Doetsch et al., 1999). Undoubtedly, astrocytes have numerous supportive functions to nervous tissue.

Because astrocytes play such fundamental functions in the brain, it is not surprising that a decrease in their function and viability has been linked to multiple neurological diseases. For

instance, reduced astrocyte function has been connected to schizophrenia and other major depressive disorders (Barres and Sloan, 2014). Also, a decline in astrocyte function has been linked to many neurodegenerative diseases. In one example, Alzheimer's disease, astrocyte function is diminished by the accumulation of amyloid- β plaques, which eventually leads to astrocyte cell death (Maragakis and Rothstein, 2006). In addition, Amyotrophic Lateral Sclerosis (ALS) is associated with the loss of an astrocyte glutamate transporter, and Huntington's disease is linked to the alteration of astrocyte gap junctions. Astrocyte deficiency has also been associated with both Alexander disease and Parkinson's disease (Maragakis and Rothstein, 2006).

Noticeably, astrocytes play an important role in the neural functioning as evidenced by the quantity of astrocytes in the CNS, the critical functions astrocytes contribute to neurons, and the significant association of deteriorating astrocytes in neurodegenerative diseases. With this in mind, it was of interest to study biological components that could increase or decrease the viability of astrocytes. One such biological component of interest is the effect estrogen has on astrocytes, and in particular, the effect that estrogen has on the oxidative stress levels in astrocytes.

Estrogen's effects on the brain are of interest for two major reasons. First, estrogen's effects on the brain are not yet completely understood, and second, estrogen is commonly prescribed in hormone replacement therapy to reduce the symptoms of menopause in women. According to the Mayo Clinic, menopause is a biological transformation period in women characterized by decreased estrogen levels and is identified with an ending of both the menstrual cycle and fertility. The progression into menopause is frequently associated with numerous uncomfortable symptoms including hot flashes, sleeping problems, fatigue, mood changes, and

increased urogenital sensitivity and dryness (Mehra 2014). To lessen these uncomfortable symptoms, many women elect to take estrogen replacement therapy as a practical remedy. The Mayo Clinic defines hormone replacement therapy as the addition of supplemental, pharmaceutically produced hormones to replenish the natural hormones that the body no longer naturally produces. However, the use of estrogen replacement therapy has been correlated with both health benefits and detriments in different studies. Because estrogen's effects on the brain are not completely understood, the use of estrogen replacement therapy is controversial. The controversy and contrasting results could conceivably stem from the type of estrogen used in therapy, rather than all estrogen therapy.

Currently, menopausal symptoms are reduced through the use of Premarin, a drug produced by Pfizer pharmaceutical company for estrogen replacement therapy. Premarin is composed of equine estrogens, derived from pregnant mare urine. Specifically, Premarin is comprised of two types of conjugated equine estrogens (CEEs), which include equilin and equilenin. However, these equine estrogens are not naturally occurring in humans and the effects of CEEs are largely unknown. There have been contending views in the literature regarding the effects of CEEs. For instance, in healthy women, CEEs have been reported to increase the risk of ischemic stroke (Hendrix et al. 2006). However, the combination of multiple CEEs has also been shown to have neuroprotective effects (Zhao and Brinton, 2006). Ultimately, the use of CEEs is currently controversial as it is unknown if the effects are beneficial or detrimental to women.

While CEEs are not naturally occurring in humans, there are three types of estrogens that naturally occur in humans. These endogenous human estrogens include estrone (E1), estriol (E3), and the most common, estradiol (E2). One particular isomer of estradiol, 17 beta-estradiol (E2), has been associated with neuroprotective and cardioprotective properties. Specifically, 17 beta-

estradiol has been found to be neuroprotective in multiple ways. First, 17 beta-estradiol has been shown to have apparent antioxidant properties that work to reduce oxidative stress in the brain (Behl et al. 1997). Also, 17 beta-estradiol has also been revealed to protect the brain, and specifically protect astrocytes, from ischemic injuries. This is thought to be due to 17 beta-estradiol's ability to reduce the harmful effects of oxygen and glucose deprivation (Guo et al., 2012). One other estradiol isomer, 17 alpha-estradiol, has also displayed neuroprotective effects similar to those of 17 beta-estradiol. In particular, 17 alpha-estradiol is effective in reducing symptoms in both Alzheimer's and Parkinson's disease as well showing subsequent usefulness following a stroke (Dykens et al., 2005). Moreover, the 17 alpha-estradiol isomer has even been shown to increase neurogenesis and positively influence cell proliferation in a dose-dependent manner (Barha et al., 2009). In essence, both 17 beta-estradiol and 17 alpha-estradiol have demonstrated neuroprotective benefits.

The purpose of this study was to discover if different types of estrogens have either a protective or detrimental effect on astrocyte cells that are exposed to oxidative stressful conditions. Astrocytes were pretreated with estrogen for one hour, subsequently exposed to oxidative stress, and then finally retreated with estrogen for two hours. These relatively shorter estrogen treatments were chosen in order to determine if estrogen has an effect via a rapid signaling pathway or a slower signaling pathway influencing transcription or translation. A transcriptional or translational change would require a longer estrogen exposure time (possibly between 24 – 48 hours) for estrogen to show an effect on astrocytes.

Also in this study, astrocytes were treated with either endogenous estrogens, which are naturally occurring in humans or CEEs, which are not naturally occurring in humans, but are often prescribed to post-menopausal women to combat the symptoms of menopause. This

allowed us to discover if naturally occurring estrogens have similar or different effects when compared with CEEs. The two endogenous human estrogens tested included: 17 alpha-estradiol, 17 beta-estradiol and the two conjugated equine estrogens tested included: equilin, and equilenin. The four estrogens were also assessed at four different concentrations between 10nM –10 μ M. This allowed us to determine whether the estrogen concentration had an effect on oxidative stress levels in astrocytes.

METHODS

Cells

In this study, we used GIBCO® Human Astrocytes (N1 1321), which are a human-based astrocyte cell line sold by Life Technologies. The human astrocytes arrived cryopreserved in liquid nitrogen. The initial portion of the experiment entailed preparing the culture of astrocyte cells. The astrocytes were thawed in a water bath at 37°C. The cells were then placed in a T-75 flask culture vessel with 10 microliters Dulbecco's Modified Eagle's Medium (DMEM), a complete astrocyte medium which also contained 10% Fetal Bovine Serum (FBS) and a 1% antibiotic/antimycotic that was supplemented to the astrocyte media. The media was changed approximately every two days. The culture vessel was incubated at 37°C in an atmosphere containing 5% CO₂. Incubation continued until cells became roughly 80-90% confluent, which was determined using a microscope. Once confluent, the astrocytes were subcultured using 5 microliters trypsin-EDTA to free cells from the substrate. Next, using a Bio-Rad automated cell counter, the concentration of cells in solution was adjusted to 1x10⁶ cells/mL. Finally, 200 microliters of the astrocyte solution was placed in 34 wells of a 96 well plate. Table 1 summarizes the treatment design for different astrocyte populations.

Astrocyte Treatment

The second portion of the experiment entailed pre-treating astrocytes, *in vitro*, with the four hormones: 17 alpha-estradiol, 17 beta-estradiol, equilin, and equilenin. The astrocytes were pretreated with 10 microliters of the estrogens for a final concentration of 10nM, 100nM, 1μM, or 10μM. There was also a positive and negative control group of astrocytes, which were not pre-treated with any estrogen. Next, half of the astrocytes were exposed to oxidative stress for one

hour by adding 20 microliters hydrogen peroxide for a final concentration of 600 μ M. The other half of the wells received 20 microliters PBS as a control. After one hour, all liquid was removed and 200 microliters of fresh astrocyte media was added. The same astrocytes, which were pretreated with the four estrogen concentrations earlier, were then again retreated with 10 microliters of their respective estrogen solutions and incubated for two more hours.

Oxidative Stress Assay

The last aspect of the experiment involved testing the astrocytes for oxidative stress using flow cytometry on the Muse® system. To accomplish this, astrocytes were first removed from each well by adding 100 microliters of trypsin-EDTA per well. Once removed, the astrocytes were placed in centrifuge tubes containing 100 microliters of fresh media and centrifuged at 3000 rpm for three minutes. Next, all liquid was removed from the centrifuge tubes, and the astrocytes were resuspended in 100 microliters of Muse® assay buffer. Then 10 microliters of that resuspended cellular solution was added to 190 microliters of prepared Muse® working solution, which allows the Muse® system flow cytometry assay to detect any reactive oxygen species. Finally, the prepared working solution, now containing the astrocytes, was incubated for thirty more minutes, and then each sample was tested via an oxidative stress assay on the Muse® system using flow cytometry.

Statistical Analysis

The independent variables were +/- hydrogen peroxide treatment and the concentration of estrogen. In total, there were five trials. Statistical significance of oxidative stress and estrogen concentration was assessed, for each estrogen, using a two-way ANOVA and LSD post hoc test. A p-value ≤ 0.05 indicated statistical significance.

RESULTS

There was not a significant difference between the positive control of astrocytes stressed with hydrogen peroxide and the negative control of unstressed astrocytes (1-way ANOVA, Stressed, $F_{1,8}=0.508$, $P=0.496$; Figure 1). The 17 alpha-estradiol assay did show a statistically significant difference when comparing estrogen treatment. However, there was no statistical significant difference across the four concentrations of 17 alpha-estradiol used (2-way ANOVA, Stressed, $F_{1,32}=7.670$, $P=0.009$; Concentration, $F_{3,32}=0.046$, $P=0.987$; Figure 2). Next, in the 17 beta-estradiol assay, there was not a statistically significant difference between the stressed and unstressed cells, and there was also no statistical significant difference across the four concentrations of 17 beta-estradiol used (2-Way ANOVA, Stressed, $F_{1,32}=3.001$, $P=0.093$; Concentration, $F_{3,32}=1.863$, $P=0.156$; Figure 3). In the equilin assay, there was no statistical significance between oxidative stress levels of the stressed and unstressed cells, and there was not a statistical significance across the four concentrations of equilin used (2-way ANOVA, Stressed, $F_{1,32}=0.067$, $P=0.797$; Concentration, $F_{3,32}=0.148$, $P=0.930$; Figure 4). Finally, in the equilenin assay, there was again no statistical significance between the stressed and unstressed cells, and there was not a statistical significance among the four concentrations of equilenin used. (2-way ANOVA, Stressed, $F_{1,32}=1.147$, $P=0.292$; Concentration, $F_{3,32}=1.417$, $P=0.256$; Figure 5).

DISCUSSION

In summary, the results showed no significant difference across the four different estrogen concentrations utilized, between 10nM-10 μ M. In addition, the results showed only the 17 alpha-estradiol assay had a statistically significant difference in oxidative stress levels across the different estrogen concentrations when comparing astrocytes which received a 600 μ M hydrogen peroxide treatment and astrocytes which did not receive any hydrogen peroxide. This may indicate that there may be an additive negative interaction when combining 17 alpha-estradiol and hydrogen peroxide. Future studies should test to see if this is valid. Finally, there was no significant difference between the oxidative stress levels of the positive control, in which astrocytes were exposed to hydrogen peroxide, and the negative control, in which astrocytes were not exposed to hydrogen peroxide.

The absence of a significant difference between the positive and negative controls could be the result of a couple factors. First, it is possible the one hour hydrogen peroxide exposure time was not long enough to significantly stress the astrocytes. It is also possible the 600 μ M concentration is not concentrated enough to induce stress to the astrocytes. However, this seems unlikely as a previous study by Grimes and Hughes showed a one hour treatment with 600 μ M hydrogen peroxide to successfully stress astrocytes (2015). Therefore, it is more likely that another factor is responsible for the inconsistency. Specifically, it is hypothesized that trypsin is responsible for the discrepancy. Trypsin is a proteolytic enzyme used to lyse cadherin molecules involved in cell adhesion (Descargues et al., 2006). Therefore, in this study, trypsin was used to facilitate the dissociation of astrocytes both from other astrocytes as well as the culture substrate. However, trypsin has been shown to lyse other cell surface proteins, which eventually leads to

the deterioration of cells (Huang et al., 2010). Thus, it is thought that trypsin, itself, is stressful to cells and could induce oxidative stress in astrocytes. Unfortunately, the trypsin exposure time was not as tightly controlled as was hoped, and different trials required different trypsin exposure periods for dissociation and eventual removal of the astrocytes. In future studies, a different removal method is recommended, if possible, or a very tight control of trypsin exposure.

Seeing as there was not a significant difference in oxidative stress levels between the positive and negative controls, it is still largely unknown if short term estrogen exposures have protective effects on astrocytes exposed to oxidative stressful conditions. However, even with the external oxidative stress induction being unsuccessful, the results still suggest one meaningful conclusion. Even in those relatively unstressed astrocytes, which were not exposed to any purposeful induction of oxidative stress, there is always some natural oxidative stress present. Therefore, the results indicate that modifying estrogen concentrations has no effect on the oxidative stress levels in the unstressed astrocyte populations. Accordingly, it is possible that estrogen does not have an effect on oxidative stress with a relatively shorter treatment time of one or two hours.

Assuming that estrogen did not have an effect on oxidative stress levels in astrocytes, it is possible that estrogen is neuroprotective to astrocytes for alternative reasons. One possible alternative is that estrogen is neuroprotective by reducing apoptosis in astrocytes. One such study has shown estradiol to have this capability by upregulating expression of the bcl-2 gene, a gene known to decrease apoptosis, especially after ischemic injuries (Dubal et al., 1999). This is further supported by Grimes and Hughes, in which they showed both 17 beta-estradiol and equine estrogens to be effective in reducing hydrogen peroxide induced apoptosis. Although, in that study, the astrocyte cell line originated from mouse cerebellum (C8-D1A), instead of the

human astrocyte cell line used in our study. Additionally, estrogen has also been shown to be protective by increasing expression of certain cytokine molecules. For instance, estradiol has been shown to upregulate the expression and secretion of transforming growth factor- β (TGF- β), a cytokine molecule with known neuroprotective benefits (Dhandapani et al., 2005). Finally, estrogens may be neuroprotective by upregulating expression of the glutamine synthetase gene in astrocytes (Mong and Blutstein, 2006). This gene is critical in astrocytes as it increases glutamine production, which is necessary for neuronal survival (Mong and Blutstein, 2006). Therefore, there are a number of alternative mechanisms, besides reducing oxidative stress, in which estrogens are thought to protect astrocytes.

Finally, it is of relevance to discuss estrogen's possible signaling pathways. Estrogen, a lipid steroid molecule, follows one of two possible signaling pathways after it diffuses into the cell. First, there is a non-genomic signaling pathway, in which estrogen binds to the G protein-coupled receptor, GPR30, inducing a release of calcium ions from the endoplasmic reticulum (Weatherman, 2006). By employing the one hour estrogen pretreatment, we sought to discover if estrogen is neuroprotective via some rapid signaling pathway, such as this. However, it is also possible that estrogen is neuroprotective via a genomic signaling pathway. For instance, in an alternative signaling pathway estrogen has been shown to influence ligand activated transcription by binding to the ER α and ER β receptors inside the cell (Weatherman, 2006). These transcriptional effects require more time and thus would not show up with the one hour estrogen pretreatment. Considering this, further inquiry into the effects of longer estrogen treatment times should be explored, such as a 24-48 hour pretreatment with estrogen. Altogether, more research is required to elucidate estrogen's effects on astrocyte cells.

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Table 1. The treatment design depicting the 34 different populations of astrocytes cultured in a 96 well plate. Each estrogen was tested at four concentrations: 10nM, 100nM, 1 μ M, 10 μ M. One half of the astrocytes were exposed to 600 μ M H₂O₂. Finally, there were two control groups without any estrogen. There was one negative control astrocyte population without H₂O₂ exposure, and another positive control astrocyte population that was exposed to H₂O₂.

Treatment	Estrogen Concentration				
17 α-Estradiol (-H₂O₂)	(-) Control (no estrogen)	10nM	100nM	1 μ M	10 μ M
17-β Estradiol (-H₂O₂)		10nM	100nM	1 μ M	10 μ M
Equilin (-H₂O₂)		10nM	100nM	1 μ M	10 μ M
Equilenin (-H₂O₂)		10nM	100nM	1 μ M	10 μ M
17 α-Estradiol (+H₂O₂)	(+)	Control (no estrogen)	10nM	100nM	1 μ M
17-β Estradiol (+H₂O₂)		10nM	100nM	1 μ M	10 μ M
Equilin (+H₂O₂)		10nM	100nM	1 μ M	10 μ M
Equilenin (+H₂O₂)		10nM	100nM	1 μ M	10 μ M

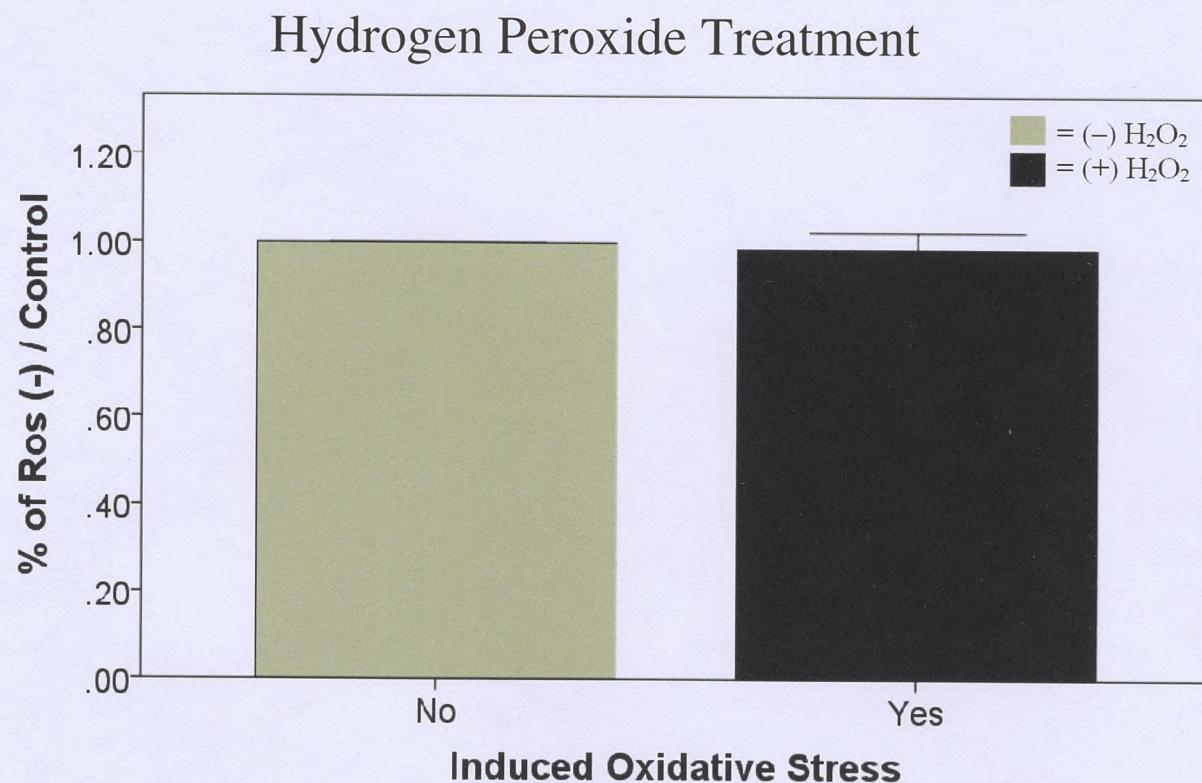


Figure 4. The average (+/- 1 S.E.) percentage of ROS (-) astrocytes of the negative control (left) and the positive control (right) when compared to the unstressed negative control.

17 Alpha-Estradiol

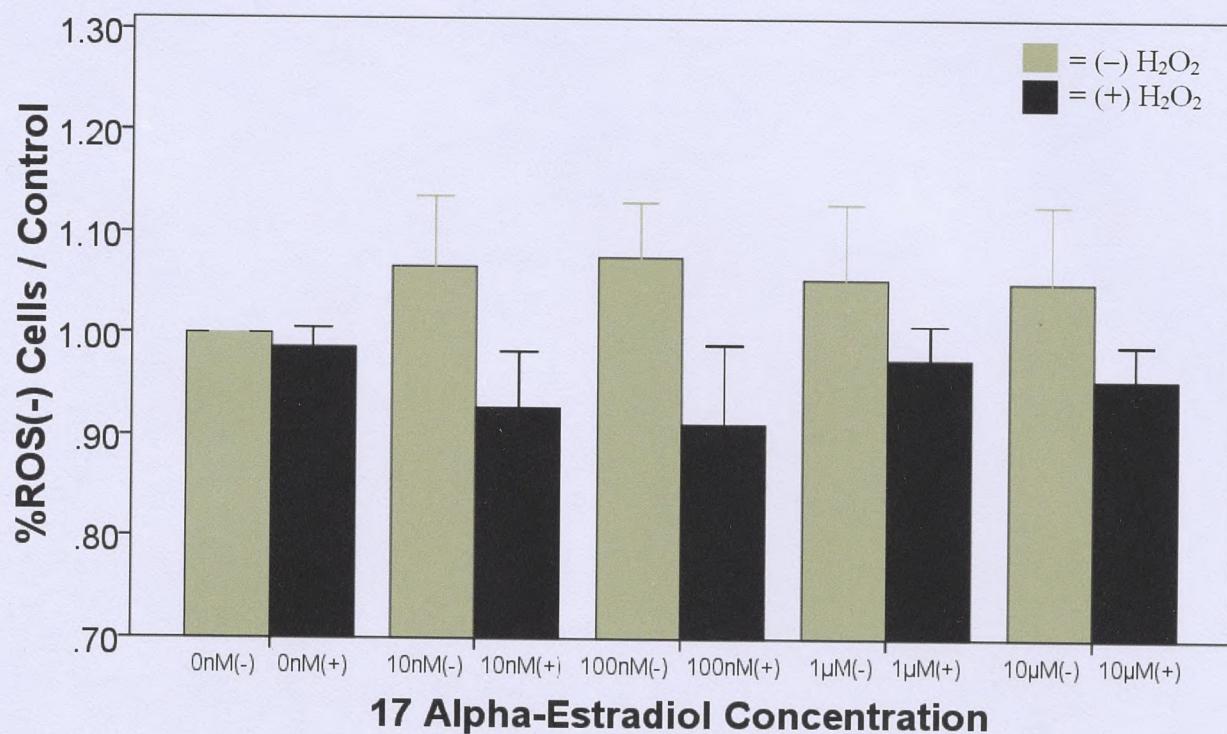


Figure 2. The average (+/- 1 S.E.) percentage of ROS (-) astrocytes compared to unstressed controls across four concentrations of 17 alpha-estradiol.

17 Beta-Estradiol

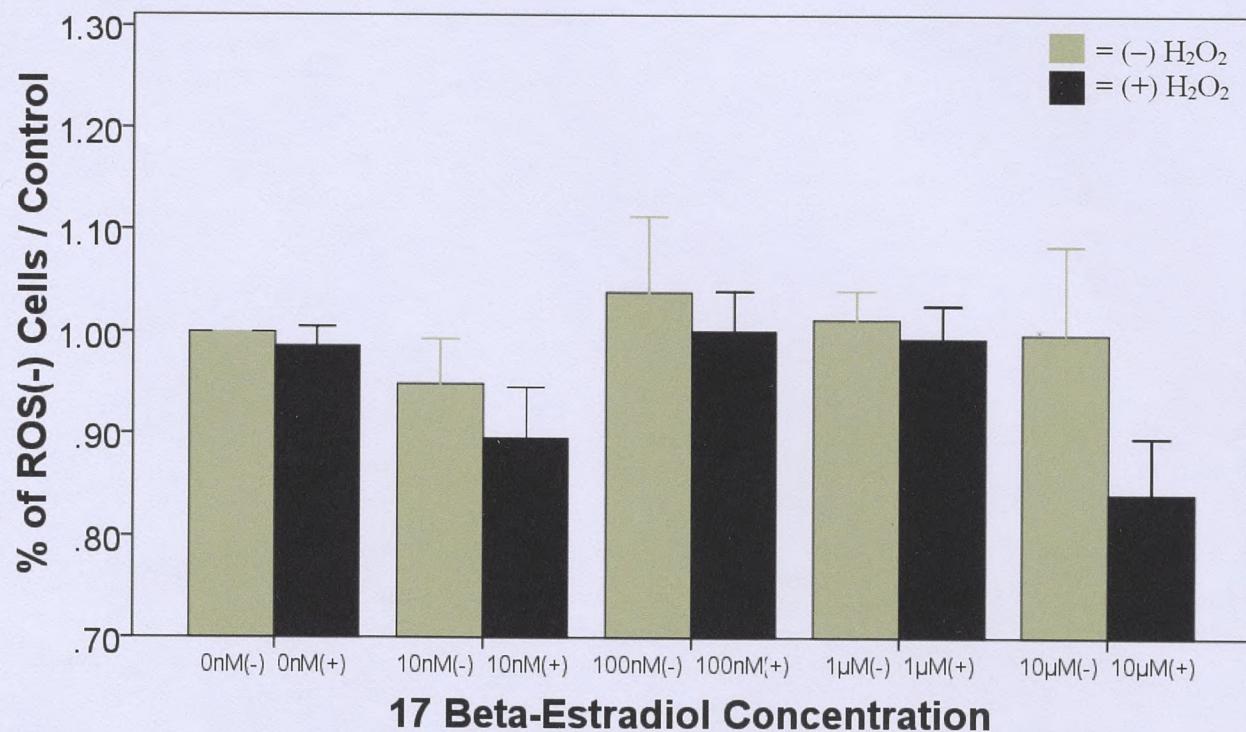


Figure 3. The average (+/- 1 S.E.) percentage of ROS (-) astrocytes compared to unstressed controls across four concentrations of 17 beta-estradiol.

Equilin

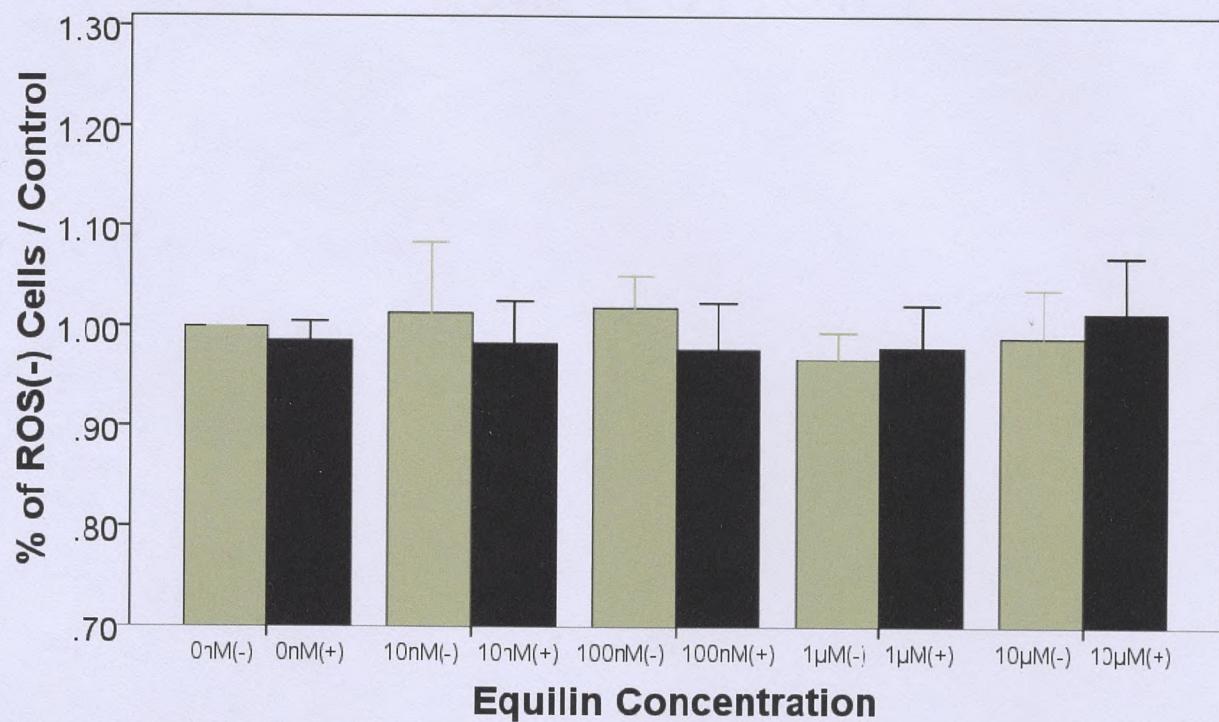


Figure 4. The average (+/- 1 S.E.) percentage of ROS (-) astrocytes compared to unstressed controls across four concentrations of equilin.

Equilenin

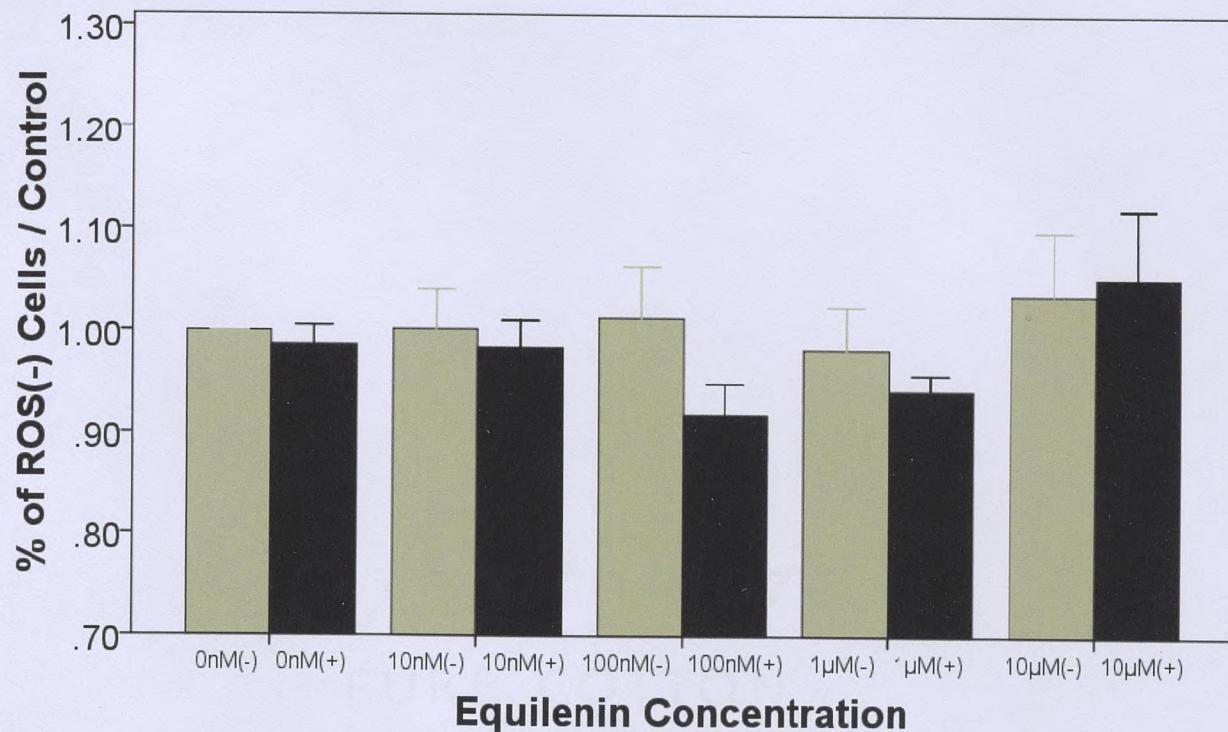


Figure 5. The average (+/- 1 S.E.) percentage of ROS (-) astrocytes compared to unstressed controls across four concentrations of equilenin.

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